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Iron homeostasis and osmoadaptation: identification of the direct regulon of Fur metalloregulators in *Chromohalobacter salexigens*

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Abstract

Fur1 and Fur2, both members of the FUR superfamily, are key transcriptional regulators found in the halophilic bacterium *Chromohalobacter salexigens*. Previous research has unveiled their pivotal roles in governing iron homeostasis. Additionally, they have been found to play a critical role in osmoadaptation, regulating the synthesis of compatible solutes such as ectoine and hydroxyectoine. In this study, we aimed to delve deeper into the roles of these transcription factors in these processes by conducting a ChIP-seq analysis of Fur1 and Fur2, elucidating their direct regulon at 0.6 and 2.5M NaCl. Our findings revealed that these transcription factors bind primarily to promoting regions, with binding motifs exhibiting similarities to known Fur motifs in other bacterial species. A striking divergence emerged when comparing the genes regulated by Fur1 and Fur2 under varying salinity conditions. Fur2 emerged as a global regulator, governing a substantial gene set across different salinity conditions, opposite to Fur1 which regulates a smaller gene set and shifts its target genes in response to salinity changes. Moreover, when comparing the genes directly regulated by both transcription factors in our ChIP-seq results with previous RNA-seq studies in Fur1 and Fur2 mutants, it became apparent that the majority of differentially expressed genes for both paralogs under different salinities showed upregulation in the Fur1 mutant and downregulation in the Fur2 mutant. Both Fur paralogs were directly associated with genes involved in siderophore biosynthesis and complex iron transport systems like ZupT and EfeUOB/M, an occurrence that becomes particularly prominent at low salinities. Finally, we found the *ectABC* cluster of genes directly related with ectoine biosynthesis, was under direct regulation of Fur2 at low salinity conditions. Taken together, our results provide novel insights on the regulatory behaviors of Fur 1 and Fur2 and their roles in iron homeostasis and osmoadaptation.

1 Introduction

Chromohalobacter salexigens is a moderately halophilic gammaproteobacterium with exceptional salt tolerance, being able to grow at 37ºC with 0.5 to 3 M NaCl in a minimal medium, and from 0.15 to 4.3 M in a complex medium [1,2]. In order to endure hyperosmotic stress and supraoptimal temperatures, *C. salexigens* adjusts its compatible cytoplasmatic solute pool, a process ruled by the uptake of external osmoprotectants or by synthesis de novo of endogenous solutes from different carbon sources [3].

Among the compatible solutes synthesized by the bacteria, ectoine and hydroxyectoine are particularly remarkable. The first one is a cyclic derivative of aspartate semialdehyde, while the second is its hydroxylated derivative [4]. The genes associated with the biosynthesis of these molecules are the gene cluster *ectABC* for ectoine and *ectD* and *ectE* for hydroxyectoine. Upregulation of these genes occurs in response to an increase in in salinity levels. Disrupting the expression of these genes could render halophiles susceptible to high salinity conditions [6]. However, this process and its regulation is quite complex, involving transcriptional and post-transcriptional processes and several global and specific regulators [3]. These molecules have an important role as osmotic protective agents while also serving as carbon and nitrogen energy sources and potential drugs for diseases such as Alzheimer's [3,7], making them highly valuable for their pharmaceutical, biotechnological, agricultural and biomedical applications. *C. salexigens's* ectoine production, ability to grow on a wide range of salinities and with a wide range of different carbon sources are some of the many key aspects that turn this bacterium into a model microorganism of maximum interest and applicability in microbial biotechnology [3,8].

Iron homeostasis has been recognized as a pivotal factor for salinity osmoadaptations in *C. salexigens*, and by extent, for the synthesis of the compatible solutes [8,9]. Iron holds paramount importance in bacterial physiology, as it functions as a cofactor of various important enzymes. To ensure the continuous production of iron-utilizing proteins, it is essential to maintain an adequate iron pool [10]. Bacteria have evolved into multiple mechanisms to regulate iron uptake when faced with limited iron availability. However, high iron levels are toxic and can lead to the generation of very reactive free radicals, underscoring the significance of iron homeostasis as a crucial mechanism that is tightly regulated in response to external iron availability [10]. The iron demand of *C. salexigens* is more pronounced at low salinity levels, a condition that correlates with an increased induction of genes involved in the biosynthesis of siderophores and genes encoding transport systems for them [8,9]. As salinity increases, the iron demand of *C. salexigens* decreases, a phenomenon caused by a decrease in protein synthesis under saline stress conditions, as many of these proteins have iron at their active site [8]. Moreover, iron homeostasis also plays a role in ectoine synthesis in *C. salexigens* since excess of iron has been reported to negatively affect the expression of the *ectA* ectoine synthesis gene [6] while it induces the *ectD* hydroxyectoine synthesis gene as a way to respond to the oxidative stress the excess iron imposes [5,8,9].

Among the global regulators described in bacteria, the ferric uptake regulator (Fur) is renowned for its role in controlling genes related to siderophore synthesis, iron transport, cytoplasmic proteins involved in metabolism and proteins combating oxidative stress [11]. Fur is a histidine-rich protein that plays a key role in maintaining iron homeostasis as global regulator in many bacteria. It primarily acts as a negative regulator when iron intracellular levels are sufficiently high by binding to Fur-box

sequences located in iron-regulated genes. It also functions as a positive regulator, but this role is less frequent, and it is mainly indirect [8, 11]. In the case of *C. salexigens,* it possesses two paralogs belonging to the FUR superfamily, namely Fur1 and Fur2 [12], both playing a crucial role in regulating iron homeostasis and osmoadaptation [8]. Transcriptional analysis based on RNA sequencing (RNA-seq) analyses on *C. salexigens* have revealed that these transcriptional factors (TFs) also play a role in metabolic and physiological adaptations to the environmental osmolarity [9]. They also exert control over the transcriptional synthesis of ectoines by upregulation of the *ectABC* gene cluster, acting as either activators or inhibitors depending on the extracellular salinity, temperature, and iron concentration. However, if it is due to a direct or indirect regulatory mechanism remains unknown [8,9]. The importance of the interplay among these interconnected processes is fundamental for unraveling the molecular mechanisms underlying the robustness and resilience of this bacterium.

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) is a central method in epigenomic research, allowing the identification of protein-DNA binding *sites in vivo,* and it is widely used to map genome-wide binding of many bacterial transcription factors [13]. In this study, we treated and analyzed ChIP-seq data from *C. salexigens* to elucidate the direct regulon of both Fur1 and Fur2 under different salinity conditions, which are conditions of low and high production of ectoines respectively.

Prior investigations through RNA-seq analyses on Fur1 and Fur2 had already provided strong indications of the essential roles played by these paralogues in processes associated with iron homeostasis and the synthesis of ectoine and hydroxyectoine, among others [12]. Our ChIP-seq analysis served as a dedicated effort to delve deeper into the direct implications of both transcription factors in regulating these crucial processes. We examined the functionalities of the genes directly regulated by Fur1 and Fur2, which exhibited variations between them and across contrasting salinity conditions. Nevertheless, our findings strongly suggested a direct involvement for both regulators at low and high salinity in iron homeostasis as well as ectoine biosynthesis, oxidative stress, energy generation, among other biological processes. Additionally, we identified potential organism-specific binding site motifs for both TFs with high statistical significance.

2 Materials and Methods

ChIP-seq data

Previous ChIP-seq assays were carried out in order to detect the direct regulon of the metalloregulators Fur1 and Fur2 of *C. salexigens* at two salinity conditions. Briefly, strains were grown in minimal medium M63 with 20 mM of glucose as sole carbon source at 0.6 M of NaCl and 2.5 M of NaCl until mid-log phase. After the crosslinking and chromatine fragmentation, AP and INPUT DNA samples were obtained by submitting the chromatine samples to affinity chromatography or not. In AP samples TF-DNA was retained in the column and submitted to a reversion of the crosslinking to elute de DNA whereas INPUT samples were directly submitted to the reversion of the crosslinking. Isolated DNA was then subjected to Illumina Next Generation Sequencing (NGS) technology, obtaining 12 FASTQ files. Specifically, eight of the samples (four AP and four INPUT) were obtained from knockout strains of Fur1 and Fur2 transcription factors grown at both salinities, where each TF was Strep-Tactin tagged and expressed by a plasmid-based specific genetic construction. Sequenced AP samples (in our case, “Affinity Pull-Down”) were used to determine the genomic regions where the TFs bind at each salinity condition [5]. “Input” samples were not subjected to affinity Chomatography immunoprecipitation so there was no DNA enrichment during the sequencing analysis. “Input”

samples represent the total fraction of DNA present in the sample and was used as a reference point for comparison with AP samples in the ChIP-seq analysis serving as controls for each specific condition [5]. Additional four samples (two AP and two INPUT) were obtained as a second negative control from cultures of *C. salexigens* wild-type strain grown at the same conditions.

Quality and quantity evaluation of ChIP-seq reads

The sequenced data was studied using linux in search for read quantity. Additionally, quality control was conducted for each sample, employing the FastQC tool [14] to scrutinize the read quality and determine whether any filtering procedures were warranted.

Alignment to the reference genome and obtention of BAM files

The reference genome "*Chromohalobacter salexigens* DSM 3043 str. DSM3043 (GCA_000055785)" in FASTA format was retrieved from the Ensembl Bacteria database [15]. This genome served as a blueprint to identify binding sites, which were subsequently assessed for their biological significance. In addition, we retrieved the corresponding GTF file, containing essential genome annotations and providing information on genes, transcripts, and precise genomic coordinates. both files were last modified as of the 11-04-2023, ensuring the use of the most current genomic data as of this date.

Upon successful retrieval of all files and quality assessment of the reads, the paired end reads from each sample were aligned to the reference genome using the paired end mode of the bowtie2 alignment tool [16], resulting in the obtention of sequence alignment map (SAM) files. The SAM files for each sample were converted into binary format (BAM), making them more space efficient for storage and faster to process. This conversion process also included the important steps of indexing and sorting the alignment data, which were carried out using SAMtools [17].

An additional analysis involving subsampling the ChIP-seq FASTA files was also carried out with the use of Seqtk (https://github.com/lh3/seqtk). A random seed of 66 was set in order to make the results reproducible and a total of 250.000 and 1 million reads were randomly selected from the files.

Peak calling

MACS2 was employed to identify and analyze significant peaks within the processed data, representing the TFs binding sites throughout the genome. This program utilizes a model-based approach to identify significant peaks in the data, estimating the background noise level and applying statistical models statistical models that employ the Poisson distribution to assess the significance of read counts within sliding windows [18].

The default model was employed for Fur1 BAM files, which is appropriate for data where fragment sizes are well defined and can be accurately modeled. This model requires estimating the fragment size distribution to accurately call peaks. In this case, the effective genome size for *C. salexigens* was set to 37 million base pairs ($3.7e^6$ bp) [19].

On the other hand, Fur2 files were treated using skipping the model building step in order to reduce noise. For this analysis, a commonly used peak size of 147 base pairs was applied. The use of '--nomodel' results beneficial when the fragment sizes in a dataset are not well defined or are highly variable. Establishing a fixed peak size can result in a more robust analysis in certain scenarios.

Visualization of ChIP-seq data

A visual representation of the analysis was performed with the use of the IGV (Integrative Genomics Viewer) program [20] to assess the significance of the generated peaks and identify potential false positives or errors during the analysis. This step involved using the *C. salexigens* genome in FASTA format retrieved from Ensembl Bacteria with its complementary GTF file, as well as the BAM and BAM.BAI files generated with bowtie2 for each TF in both 0.6M and 2.5M NaCl conditions. The narrow.peak and BED files with the information of the peaks and summits information identified with MACS2 were also utilized as they are the most crucial in this analysis to discern the TF binding locations.

Motif discovery

Leveraging the Galaxy community [21] online platform, the nucleotide sequences from the BED files containing peaks generated during the macs2 analysis for both Fur1 and Fur2 were identified and transcribed into four FASTA files using the "getfasta" tool from the BEDTools program [22].

MEME-ChIP from the MEME suite of software [23] was used to further elucidate potential motifs within these sequences. The "Classic mode" was selected for individual motif discovery within each FASTA file.

MEME-ChIP also carried out a motif enrichment analysis. The CollectTF database, which exclusively harbors experimentally validated bacterial TF binding sites [24], was selected among the options offered by the program to find matches with the input data. The number of motifs set to find was set to 5 with default settings.

ChIP-seq peaks association with genes

The GTF file retrieved from the Ensembl Bacteria database served as a foundation for constructing a local database in the form of a TxDB object (transcript database) using tools from the R library GenomicFeatures [25]. Subsequently, R libraries ChIPseeker [26] and GenomicRanges [25] were employed to record information of the promoter regions within the *C. salexigens* genome.

The collected information was used for annotating the peaks from the narrow.peak and BED files based on their proximity to a transcription start site (TSS). Annotations were filtered, retaining upstream gene regulators, aligning with the focus of this study on the direct regulon of both Fur1 and Fur2 transcription factors.

Gene-operon association

A dataset previously acquired by the research group containing all the operons for the *C. salexigens* genome, was sourced from the Prokaryotic Operon Database (ProOpDB) [27]. We used this dataset to reveal the entire set of genes directly regulated by Fur 1 and Fur2 at both 0.6 and 2.5 M NaCl. For this matter, we converted the dataset into CSV format and imported it to R. Following this step, we conducted an intersection between this dataset and the annotated genes assigned to ChIP-seq peaks for both Fur1 and Fur2, using the same tool, allowing the addition of the genes that were part of the same operon as those initially detected after peak calling into the set of genes regulated by Fur1 and Fur2. This process resulted in the obtention of the complete direct regulon for each regulator at both 0.6 and 2.5M NaCl.

Gene annotations

A dataset previously acquired by the research group contained valuable annotations for the *C. salexigens* genome, such as GO terms and their accession numbers, KOs, NCBI and KEGG was employed to associate valuable information of Fur1 and Fur2's direct regulon. The dataset was converted into CSV format and uploaded to R. From then, we performed an intersection between this dataset and the complete set of genes under direct regulation of Fur1 and Fur2. This process facilitated the addition of functional information to each of the genes in the complete set directly regulated by Fur1 and Fur2 under both salinity conditions.

Functional enrichment analysis

Gene IDs for all the genes regulated by Fur1 and Fur2 at 0.6 and 2.5M NaCl were employed to identify enriched biological processes and pathways using ShinyGO [28]. For this process, we utilized the gene IDs from Fur1 and Fur's sets of regulated genes under both 0.6 and 2.5M salinity conditions as inputs. The analysis was conducted using the default parameters for the online tool, and the resulting bar plots displaying enriched biological processes and pathways were downloaded for further examination.

Correlation of ChIP-seq Data with Expression Data

A previous RNA- Seq analysis conducted by Naranjo [12] laid the foundation for a valuable cross-reference with the ChIP-seq results in order to validate the outcomes of the of the ChIP-seq bioinformatic analysis, also enabling the exploration and dentification of patterns within the posed data.

An intersection of the RNA-seq and ChIP-seq datasets was carried out using R. The combined dataset underwent a filtering process, focusing on genes that exhibited significant FoldChange values falling within the range of greater than 1.8 and less than -1.8, along with a pValue of 0.1 or lower. The genes present in both datasets were categorized into two groups based on their positive or negative FoldChange values, enabling us to associate the activator or inhibitor roles of Fur1 and Fur2 on these genes.

3 Results and Discussions

3.1 Data processing and filtering

The ChIP-seq sampled data (AP and INPUT) of Fur1 and Fur2 and wild type control at low and high salinity conditions obtained from the NGS sequencing averaged between 20-30 million reads per sample, providing a substantial quantity of data essential for conducting a comprehensive and in-depth study. The quality control performed with FastQC exhibited exceptional quality, obviating the need for filtering.

Additional analyses for both TFs were also carried out using ChIP-seq subsampled data, reducing the number of reads to 250 thousand and 1 million. The objective was to relieve computational processing time and explore the limitations of the data. The main findings of these analyses were the increase in number of the peaks found using MACS2, most of which were also found in the BED files generated from their full dataset counterparts. However, the full dataset analysis offered more specificity, allowing for more comprehensive and accurate results. Consequently, the rest of our results were based on the complete dataset.

The high-quality reads and lack of promiscuous alignments led to mapping rates around 90% to the *C. salexigens* DSM 3043 reference genome for the majority of the reads for Fur1 and Fur2 mutant strains and wild type strains at 0.6M and 2.5M NaCl (*Table 1*). The alignment of the reads to the genome also resulted in the generation of well-defined ChIP-seq signals (**Figure 1A and B**). MACS2 was employed to identify significant peaks, setting a q-value threshold of 0.05, thus ensuring a reliable basis for peak detection.

While the Fur1 peak detection was carried out successfully using the default model of MACS2 (**Figure 1A**), Fur2 required model adjustments due to limitations posed by the data. The high read depth and variability in fragment sizes posed unique challenges for the program to effectively capture the underlying patterns and variations, resulting in an excess of significant peaks with many false positives. By bypassing the model building step and adopting a peak size of 147 base pairs, we managed to reduced noise and improve specificity, resulting in the reduction in the number of peaks, these being more concrete and visually significant (**Figure 1B**). The analyses were recreated using the same programs and parameters, utilizing the Galaxy community online platform [21], culminating with the exact replication of our results.

As the primary objective of this study was to elucidate the direct regulon governed by Fur1 and Fur2 metalloregulators, filtering of non-promoter binding sites was performed to focus the analysis on the genes and promoter regions where Fur1 and Fur2 exert direct transcriptional control.

3.4 3.2 Motif analysis

To further prove the validity of our results, we performed a motif analysis using the sequences associated with significant peaks identified by MACS2 which represent putative binding sites for Fur1 and Fur2.

For this matter, the online tool MEME-ChIP, specialized in motif discovery for ChIP-seq data was employed. It identifies motifs using two motif discovery algorithms based on probabilistic and non-probabilistic models (MEME and DREME respectively) and then performs a motif enrichment with known DNA-binding motifs [23]. For this precise analysis, the bacterial transcription factor binding site database CollectTF [24] was employed due to the lack of organism specific databases for *C. salexigens*.

Several TF motifs were found for Fur1 at both 0.6 and 2.5M NaCl using the DREAM algorithm with very low e-values. Some of the motifs found were similar to Fur motifs in other bacteria such as *P. Syringae* or *C. Crescentus.* Curiously, some of the motifs found for this TF were matched with other transcription factors other than Fur. (**Supplementary figure 1A**).

Fur2 motifs were identified using both DREAM and MEME motif discovery algorithms (**Supplementary figures 1C and 1D**). One of the identified motifs stands out due to its statistical significance, resulting in an e-value score of 6.9e-164 (**Supplementary figure 1C**). This motif was found for both 0.6 and 2.5 M NaCl conditions using both algorithms, and shares similarities with Fur motifs the same bacteria previously mentioned among others, such as *L. monocytogenes, V.cholerae* or *A.salmonicida.*

These findings demonstrate the effectiveness of our ChIP-seq analysis, as the motifs identified exhibit remarkable similarity to conserved Fur motifs in other bacteria. Interestingly, some of the enriched motifs with significant *e-values* were not matched with other similar motifs in the CollecTF

database. This could be indicative of specific binding sites for Fur or other transcription factors in *C. salexigens* (**Supplementary figures 1A, 1B and 1D**). Experimental validation should be considered to confirm these motifs' functional significance.

3.5 3.3 Identification of genes regulated by Fur1 and Fur2

Results of the bioinformatic analysis of the ChIP-seq peaks provide valuable insights into the binding patterns of both Fur1 and Fur2. The data shows evidence that these transcription factors exhibit affinity for binding primarily to promoter regions near the TSS (**Figure 2A**). In addition, we observed some binding sites located within gene-coding regions and distal intergenic regions, a phenomenon observed specifically for Fur2 (**Figure 2B**). This diversity in peak locations reinforced the importance of our peak filtering approach, which focused on retaining peaks in promoter regions to elucidate the direct regulon for both TFs.

The identification of the genes regulated by each TF was established using the ChIPseeker R package, based on the information provided by the transcript database generated from the GTF file for *C. salexigens* retrieved from Ensembl Bacteria [15]. This database contained metadata and gene annotations for the genome of the bacteria, enabling the examination of the genomic regions where significant peaks were identified. ChIPseeker utilizes a proximity base method, locating the nearest upstream or downstream gene from where each peak is located [22].

A clear difference in the number of regulated genes between both transcription factors across different salinity conditions was a focal point in this analysis. A total of 33 genes were identified for Fur1 at 0.6M NaCl, while 30 were found for the same TF at 2.5M NaCl. In the case of Fur2, an outstanding number of 368 genes were singled out for low salinity, while 84 were found for the high salinity (**Figure 1E)**. Both TFs seem to be affected by salinity but in a different manner. For Fur1, salinity could affect the binding site since it binds to different areas of the genome. For Fur2 however, it appears that salinity could affect the binding itself, implying a reduced affinity for specific promoters.

Another intriguing occurrence is the difference between the shared genes that these TFs regulate at different conditions. Fur1 exhibits a substantial shift in its target genes depending on salinity conditions, regulating an almost completely different set of genes. In contrast, Fur2 regulates a lower number of genes at high salinity compared with low salinity, but the majority of those are shared with its targets at lower salinity conditions. This could imply that the regions where Fur2 binds may not be influenced by salinity, as it maintains its affinity for them at both high and low salinity conditions (**Figure 2C**). This consistent regulatory control over a core set of genes across different salinity conditions is highlighted by our motif analysis. Specifically, we identified a motif with exceptionally high statistical significance, which was found for Fur2 at both 0.6 and 2.5M NaCl (**Supplementary figures 1C and 1D**).

These remarkable results suggest that Fur1 acts as a more specific regulator with localized effects, changing its target genes in a salinity-depending manner. In contrast, Fur2 appears to function as a global regulator, governing a higher number of genes, with a significant overlap in their regulation at both salinity conditions.

3.6 Operon inference to identify the complete direct regulons of Fur1 and Fur2

One of the primary objectives of this study was to compare the direct regulons of Fur1 and Fur2 mutants in relation to previously obtained RNA-seq data from the same mutated TFs conducted by Naranjo [12]. Our ChIP-seq analysis allowed us to identify the genes directly associated with the

binding sites of these regulators, which corresponded to the first gene of the operon. However, it became evident that many genes were not directly captured in the ChIP-seq results, since some of them are part of the operon but located in a more distant position respect to the TTS. To address this limitation, we conducted an operon inference by cross-referencing ChIP-seq identified genes related to peaks in promoter regions for both TFs at different salinity conditions with a predicted operon dataset of *C. salexigens*, previously acquired by our research group. This approach enabled us to obtain complete direct regulons of Fur1 and Fur2 at both salinity conditions.

Therefore, Fur 1 was found to regulate a total of 84 genes at 0.6M NaCl and 60 genes at 2.5M NaCl. On the other hand, Fur 2 seemed to regulate 893 and 213 genes at low and high salinity respectively.

The operon inference allowed for a wider view of the regulated genes shared by Fur1 and Fur2 at different salinity conditions. This analysis reinforced a previously observed pattern, where Fur1's regulated genes shift with contrasting salinity levels, while Fur2 maintains a more consistent set of regulated genes across varying salinity conditions. Fur1 only share 9 genes between different salinity conditions, while Fur2 share a total of 188, only varying in 25 of them out of the 213 it regulates at high salinity (**Figure 3A**). Along with these findings, we also encountered overlaps between Fur1 and Fur2 under the same salinity conditions (**Figures 3B and 3C**), suggesting they may have related or interconnected functions in response to salinity.

3.7 Functional enrichment analysis

Subsequently, the online tool ShinyGO was employed to identify overrepresented GO terms and pathways associated with the genes governed by each TF at both salinity conditions, providing insights into their functional roles and biological importance [28].

Clear differences in the pathways and biological processes regulated by Fur1 and Fur2 can be observed. These disparities extend not only between the two regulators themselves but also within the processes they oversee under different salinity conditions. In this regard, Fur1 was found to be primarily involved in biotin biosynthesis and arginine and thiamin metabolism at 0.6M NaCl (**Figure 4A**). Previous findings related with the osmoadaptation of this bacterium have demonstrated the regulation of metabolic processes such as organic acid consumption, a process that seems to be under Fur1's regulation (**Figure 4A**) [8, 29]. Another interesting finding is its involvement in zinc transport (**Figure 4A**). Zur (zinc uptake regulator) is a zinc regulator from the FUR superfamily of proteins that despite only sharing a 27% identity with Fur in *E. coli*, shares some functional traits with it [30], which may coincide with this result. On the other hand, biological process profile regulated by Fur1 was totally different at 2.5 NaCl. Metabolic processes and zinc uptake did not appear to be regulated at this condition, favoring mostly processes involving phosphate transport, asserting the TFs regulation of energy generation related processes [29] (**Figure 4B**). Other key processes regulated by this TF at high salinity are the Trap/TeaA transportation systems, which are related to ectoine and hydroxyectoine transportation among other compatible solutes and coincides with hydroxyectoine maximum production at this salinity [5, 31] (**Figure 4B**).

Our results on Fur2 revealed a greater diversity of processes and pathways directly governed by this TF. Quorum sensing, ribosomal and rRNA binding appeared consistently as enriched processes at low and high salinity, with other less enriched examples being stress response [8,10,11], TCA cycle and oxidative phosphorylation, processes induced at high salinity and correlated with higher biomass and ectoine production [9, 29, 31]. Moreover, the results also indicated involvement in solute binding and

protein export processes, and the regulation of ABC transporters, TonB boxes and permeases. All these processes align with Fur's main role in iron homeostasis, osmoadaptation and metabolic adaptations to salinity [9,12] (**Figures 4C and 4D**).

The differences in biological processes and pathways regulated by this TF are not as evident as they are in Fur1 at different salinity conditions. Some of the discrepancies are associated with processes like cell wall biosynthesis and motility, which have been observed to be induced by high salinity in this bacterium [9] (**Figure 3A**). The similarities in the processes and pathways regulated by Fur2 at 0.6 and 2.5M NaCl could once again be attributed to Fur2 regulation pattern, as it appears to be governing over a core set of genes independently of salinity.

3.8 ChIP-seq – RNA-seq correlation

A previous RNA-seq study conducted by Naranjo was performed on Fur1 and Fur2 mutants at 0.6 and 2.5 M NaCl and comparing the whole-genome expression to the wild type strain [12]. This analysis established differentially expressed genes as significant by utilizing a "fold-change" value threshold of -1.8 and 1.8 for downregulated and upregulated genes respectively and an adjusted 0.1 p-value cutoff [12]. By cross-referencing the directly regulated genes of both TFs in our ChIP-seq analysis with the ones that exhibited a significative differential expression in RNA-seq, we aimed to gain a deeper understanding on both TF's regulatory roles (*Supplementary tables 5-8*). In this comparison, the number of regulated genes identified by our ChIP-seq analysis was lower than those identified by RNA-seq, indicating that not all the genes were directly controlled by Fur1 and Fur2 exhibited a significant differential expression. Specifically, for Fur1, we found that at 0.6 M NaCl, 37 out of the 84 regulated genes identified by ChIP-seq displayed differential expression, 20 of them being upregulated and 17 downregulated (*Supplementary table 5*). At 2.5 M NaCl, out of the 60 regulated genes, 25 exhibited differential expression, with 19 being upregulated and 6 downregulated (*Supplementary table 6*). For Fur2 at 0.6 M NaCl, we observed differential expression in 404 out of the 893 regulated genes, being 167 upregulated and 236 downregulated (*Supplementary table 7*). Finally, at 2.5 M NaCl, for Fur2, 67 out of the 213 directly regulated genes showed differential expression, including 27 upregulated and 32 downregulated genes (*Supplementary table 8*).

Based on the intersection of RNA-seq data with genes regulated by Fur1 and Fur2 in our analysis, we can observe a slight majority of the significantly differentially expressed genes being upregulated for the Fur1 mutant strain (*Supplementary tables 5 and 6*) (**Figure 5**). However, an intriguing observation arises with Fur2 as most of the genes under the regulation of its mutant are downregulated. This effect is more pronounced at 0.6M NaCl (*Supplementary tables 7 and 8*) (**Figure 5**). Further analysis is warranted to explore this phenomenon in greater detail.

3.9 Fur1 and Fur2 role in iron homeostasis

A second dataset containing valuable gene annotations of the *C. salexigens* genome was previously acquired by our research group (*Supplementary table 9*). This dataset contained GO terms and their accession numbers, KOs, NCBI and KEGG annotations for each specific gene in *C. salexigens*. We integrated these comprehensive annotations with the sets of genes directly regulated by Fur1 and Fur2 under both 0.6 and 2.5 M salinity conditions. This process yielded a complete dataset with all the genes that were found to be regulated by both TFs during our ChIP-seq analysis and their respective functional annotation. This allowed for a deeper exploration of each specific gene under regulation of Fur1 and Fur2. Our results were consequentially validated with previous RNA-seq studies on *C. salexigens* under the same salinity conditions [9,12].

C. salexigens Fur direct regulon identification

While our results reveal clear differences in the genes each regulator targets, they consistently suggest that both Fur1 and Fur2 exert direct control over different genes involved in iron homeostasis, a process that is primarily mediated by Fur in many bacteria [8, 10, 11, 12]. Remarkably, Fur2 appears to regulate a larger number of genes participating in this process. In our findings, both Fur paralogs are involved in the regulation of genes responsible for the synthesis and transportation of siderophores and iron transport at both salinity conditions. The quantity of the genes related to these processes its more pronounced at low salinity for both TFs, which could correlate with *C. salexigens* higher iron demand at low salinity levels and a lesser production of siderophores when salinity levels rise [8,9].

Fur2 appears to play a more predominant role regulating siderophore biosynthesis, as its mutant regulates a gene coding for isochorismate-pyruvate hydrolase at low salinity (*Csal_1779*), which is responsible for initiating siderophore biosynthesis [9]. A gene cluster related to siderophore biosynthesis formed by a gene cluster composed of *Csal_1053, Csal_1054* and *Csal_1055* (*Supplementary table 3*) was also under direct regulation of the Fur2 at low salinity. Previous RNA-seq data of a Fur2 mutant of *C. salexigens* showed an increase of the expression of these genes under the same salinity conditions, suggesting the involvement of this Fur2 as an inhibitor [12]. Furthermore, The sigma factor σ24 Fec1, encoded by *Csal_1052* and located upstream of the mentioned gene cluster, is a transcriptional regulator associated with siderophore synthesis [4]. *Csal_1052*, is under regulatory control of the Fur2 at both low and high salinity levels (*Supplementary tables 3-4*). The overexpression of a Fur2 mutant at low salinity implies negative regulation by Fur2 for this condition [12]. At high salinity however, this gene was found to be induced [9].

In addition, we identified genes (*bfr*) encoding for iron-chelating agents such as bacterioferritin (*Csal_2899*), an important component for siderophore biosynthesis. Prior studies have established that the expression of *bfr* genes, which encode for bacterioferritin, are under the regulatory control of Fur [35]. This gene also appeared under regulation of both Fur1 and Fur2 at low salinity conditions in our ChIP-seq analysis (*Supplementary tables 1-3*). Noteworthy, this gene has been reported to be induced under low salinity for Fur1 and Fur2 mutants [12], which may suggest Fur1 and Fur2's possible roles as inhibitors for this gene.

Regarding siderophore transport, genes coding for ABC-type transport systems for siderophores such as *Csal_0551, Csal_2702* or the gene cluster *Csal_1041-1043* appear to be regulated by Fur2 (*Supplementary table 3*) at both salinity conditions. The distinction between the first two and the gene cluster lies on their upregulation under different salinity conditions: the first two at low salinity and the gene cluster at high salinity [9,12].

Genes coding for TonB siderophore receptors such as *Csal_2539, Csal_3258* and were also found to be under direct regulation of the Fur2 at low salinity (*Supplementary table 3*). These two genes appeared to be overexpressed in the Fur2 mutant, hinting for the TF role as an inhibitor [12].

Two important siderophore-independent iron transportation systems were found to be under direct regulation of Fur1 and Fur2 at low salinity. *ZupT* is part of a transportation system responsible for zinc absorption in many bacteria, but it also acts as a divalent metallic cation transporter including Fe^{+2} [36]. The *ZupT* gene (*Csal_3261*) was found to be under direct regulation of Fur2 at 0.6M NaCl (*Supplementary table 3*), confirming a phenomenon that was first reported by Naranjo by qPCR analysis, evidencing the regulatory role of Fur regulators over this transporter [12]. This not only showed the Fur2 involvement in iron homeostasis but also hints at a possible role for this TF the regulation of zinc transport, a process that was previously highlighted for Fur 1 (**Figure 2A**).

The other regulated transportation system included *Csal_3311* (*EfeM*), *Csal_3312* (deferrochetalase), *Csal_3314* and *Csal_3313* (iron transporter and iron transporter substrate-binding protein). These four genes seemed to be part of an operon that encodes the EfeUOB/M iron transporter [12]. These four genes are also preceded by *Csal_3315* and *Csal_3316* (*yidC*), coding for a GTPase and a translocase respectively (*Supplementary tables 1 and 3*). A previous RNA-seq analysis performed by Naranjo for Fur1 and Fur2 mutants showed an upregulation of this gene cluster in the Fur1 mutant but a downregulation in the Fur2 mutant under conditions of low salinity and iron limitation [12]. These findings could indicate that Fur 1 could act as an activator for this transporter system and consequently contribute to the accumulation of intracellular iron at low saline concentrations when there is no iron supplementation. In contrast, Fur2 may function a repressor for the transportation system [12].

This transportation system appeared to be under the direct regulation of both Fur1 and Fur2 at 0.6 M NaCl in our ChIP-seq analysis, comprising 4 of the 44 genes shared by both TFs (**Figure 3B**), including other iron homeostasis related genes such as the previously mentioned *Csal_2899*, coding for bacterioferritin (*Supplementary tables 1 and 3*). As salinity increased, the number of shared regulated genes by both TFs decreased to 16 (**Figure 3C**). Genes related to iron homeostasis became less prevalent. Instead, we found genes encoding for the DNA-processing chain A protein *DprA* (*Csal_2865*) and a LysR family transcriptional regulator (*Csal_1554*), and 3 genes related to oxidoreductase activity (*Csal_2579-Csal_2863*) (*Supplementary tables 2 and 4*). The regulation of these genes suggests a possible role for both TFs in oxidative stress regulation [37, 38]. This process in has been previously linked with Fur in other bacteria such as *Escherichia coli*, *Riemerella anatipestifer* or *Chromobacterium violaceum* [10, 38, 39]. These genes appeared to be differentially expressed for Fur2 and Fur1 mutants in previous studies, complementing our results [12].

3.10 Fur regulation of ectoine and hydroxyectoine synthesis

In previous qPCR studies of *C. salexigens*, it was established that Fur acts as a positive regulator of ectoine synthesis by exerting control over the *ectABC* gene cluster at high salinity [8,9]. We aimed to investigate how these findings correlate with the results of our ChIP-seq analysis on *C. salexigens*.

In our study, we found that only Fur2 was directly regulating the *ectA*, *ectB* and *ectC* genes (*Csal_1876*, *Csal_1877*, *Csal_1878*) at 0.6M NaCl (*Supplementary table 1*). These findings are in line with the results observed by Naranjo, where low salinity and iron depletion led to a 2-fold decrease in the expression of this cluster in *C. salexigens* strains in a Fur2 mutant, indicating that Fur2 could act as an activator of ectoine synthesis under such conditions [12].

The *ectD* gene (*Csal_0542*) codes for an ectoine hydroxylase, involved in the synthesis of hydroxyectoine. Similarly, a related gene, e*ctE* (*Csal_3004*), also encodes a secondary functional ectoine hydroxylase, contributing both to *C.Salexigens* thermoprotection [40]. Neither of these genes were found to be under direct regulation of Fur1 and Fur2 in our analysis. This absence could be attributed to the fact that our study did not account for high-temperature conditions, which, along with high salinity, plays a critical role in the maximal production of hydroxyectoine [5, 40].

4 Conclusions

Our study shed light on the direct regulon of Fur1 and Fur2, uncovering their direct influence on iron homeostasis and osmoadaptation. Additionally, in this work we have gained valuable information of the regulatory roles of Fur1 and Fur2 transcription factors in *C. salexigens* at different salinity conditions.

These transcription factors have a tendency to bind to promoter regions and have conserved binding motifs similar to other Fur binding motifs in other bacteria. Moreover, we discovered a few motifs with high statistical relevance that lacked prior similarities to binding motifs of other transcription factors in bacteria, which may signify their specificity for this bacterium.

Both TFs seem to respond to salinity in a different manner. In the case of Fur1, salinity appears to influence binding sites, leading to its association with different genomic regions. Fur2's response on the other hand, suggests that salinity directly affects its binding process, possibly diminishing its affinity for specific promoters. Notably, Fur2 appears to have a role as a global regulator, controlling a large set of genes, with many of these genes remaining under Fur2's control at both low and high salinity conditions. In contrast, Fur1 displayed a less global regulator role, regulating a lesser number of genes and shifting their targets as salinity rises. This difference in regulatory behavior between Fur1 and Fur2 is an intriguing occurrence, suggesting distinct roles for these transcription factors in response to salinity changes.

Additionally, the regulated genes that displayed differential expression were predominantly upregulated for Fur1 mutants and downregulated for Fur2 mutants. This observation opens possible new investigations into the mechanisms which by Fur2 modulates gene expression, especially at low salinity.

Both regulators seemed to be directly regulating genes associated with siderophore biosynthesis and transport, Fur2 exhibiting a more prominent role in their regulation. Important iron transport systems such as ZupT and EfeUOB/M were also found under Fur1 and Fur2. It is noteworthy that both Fur1 and Fur2 showed a more pronounced regulatory influence on genes related to these biological processes at low salinity conditions.

Remarkably, the *ectABC* gene cluster associated with the biosynthesis of ectoines, compounds critical for osmoadaptation in this bacterium as compatible solutes, was also found under direct regulation of Fur2 at low salinity. Our findings are consistent with prior RNA-seq studies on Fur2 mutants, suggesting that Fur2 may serve as an activator of the ectABC genes, promoting the biosynthesis of these crucial molecules at these specific salinity conditions.

These findings emphasize the significance of Fur1 and Fur2 in the context of osmoadaptation and maintaining iron homeostasis in *Chromohalobacter salexigens*.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

L.T.A. performed the necessary ChIP-seq analyses, motif analysis and comparisons to previous RNA-seq data. M.A.B. and M.S.L reviewed and supervised the project and manuscript, also providing the necessary material.

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9 Data and Code Availability Statement

Sequencing data is available upon request. Scripts used for this analysis can be accessed through the following GitHub repository:

https://github.com/Luis7orres/TMF_LuisTorres

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11 Figure legends

Figure 1. Fur1 and Fur2 generated peaks.

(A and B) Comparative example view of AP (Antibody pulldown) sample reads forming clear and visible peaks compared to input sample reads in Fur1 and Fur2.

Figure 2. Fur1 and Fur2 binding sites and genomic location. **(A)** Peak locations relative to transcription start site (TSS). The predominant binding area for both Fur1 and Fur2 at 0.6 and 2.5 are promoter regions, followed by coding regions. Distal intergenic regions were only found for Fur2 at 2.5M NaCl. **(B)** Peak profiles, representing peak count frequency across different genomic regions, demonstrating a tendency of the binding areas to be located near the TSS or TTS. **(C)** Regulated genes assigned to peaks for both Fur1 and Fur2 in different saline conditions after non-promoter binding site filtering**.**

Figure 3. Overlap in genes regulated by Fur1 and Fur2 at 0.6M and 2.5M NaCl.

(A) Complete overview of the overlap in regulated genes by Fur1 and Fur2 at 0.6M and 2.5M NaCl conditions.
(B) Overlap in regulated genes by Fur1 and Fur2 at 0.6 NaCl salinity conditions, indicating 44 genes are shared by both TFs.
(C) Overlap in regulated genes by Fur1 and Fur2 at 2.5 salinity conditions. The number of shared genes decreases as salinity rises, only 16 genes being shared by both TFs.

Figure 4. Functional enrichment for Fur 1 and 2 regulated genes at 0.6 and 2.5M NaCl

(A) Functional enrichment of genes under direct regulation of Fur1 for 0.6M NaCl conditions, asserting a clear linkage with biotin biosynthesis and regulation, several metabolic processes such as organic acid consumption and zinc transport.
(B) Functional enrichment of genes under direct regulation of Fur1 for 2.5M NaCl conditions. Pathways and biological processes associated with the genes the TF regulates at high salinity conditions shift drastically, mainly regulating phosphate transport and Trap/TeA transportation systems.
(C) Functional enrichment of genes under direct regulation of Fur2 for 0.6M NaCl conditions. Quorum sensing and rRNA and ribosomal subunit binding show a clear enrichment, followed by other important processes for Fur as regulators of iron homeostasis.
(D) Functional enrichment of genes under direct regulation of Fur2 for 2.5M NaCl conditions. The enriched biological processes and pathways almost coincide considerably with the ones for the low saline condition.

Figure 5. Gene regulation profile by Fur1 and Fur2 at 0.6 and 2.5 M NaCl.

Fur2 has a clear role as an activator for both salinity conditions as the majority of genes regulated by its mutant are downregulated, being more apparent at 0.6M NaCl. Fur1 on the other hand appears to mainly function as an inhibitor, evidenced by the majority of the genes regulated by its mutant are upregulated.

Supplementary figure 1. Significant motifs found in Fur1 and Fur2 binding sites

(A) Significant motifs found for Fur1 at 0.6M NaCl. Both motifs were found by the same algorithm. The first one show similarity with Fur motifs in other bacteria, while the second was deemed statistically significant despite not sharing similarities with other motifs, meaning it could be a specific binding motif for Fur in *C. salexigens*.
(B) Significant motif found for Fur1 at 2.5 M NaCl. Another possible specific binding motif for Fur in *C. salexigens.*
(C) Significant motifs found for Fur2 at 0.6M NaCl. The first motif has remarkable statistical significance and was found by both motif discovery algorithms. The second could be one specific binding motif for Fur in *C. salexigens*
(D) Significant motifs found for Fur2 at 2.5 M NaCl. The first motif is the same that was found for the same TF at at salinity. Both motifs share similarities with Fur motifs in other bacteria.

12 Table Headers.

Table 1. Percentage of mapped reads from C. salexigens samples

Supplementary table 1. Genes regulated by Fur1 at 0.6M NaCl

Supplementary table 2. Genes regulated by Fur1 at 2.5M NaCl

Supplementary table 3. Genes regulated by Fur2 at 0.6M NaCl

Supplementary table 4. Genes regulated by Fur2 at 2.5M NaCl

Supplementary table 5. Fur1 gene regulation profile at 0.6M NaCl

Supplementary table 6. Fur1 gene regulation profile at 2.5M NaCl

Supplementary table 7. Fur2 gene regulation profile at 0.6M NaCl

Supplementary table 8. Fur2 gene regulation profile at 2.5M NaCl

Supplementary table 9. Genome annotations for C. salexigens